PCMass25 MANUAL

1.Introduction

PCMass25.exe (i.e. 2.5) is a program written in C which runs in Windows 95 or higher for analysis of BNL STEM images. Any PC with greater than 16Mbyte memory and a 1024x768 high-color or true-color display should be able to run these programs. A processor speed of >200 MHz gives minimal delays for most operations. A good Internet connection should allow you to receive images by Internet (see FTP protocol and Tutorial #1). STEM images are 528,384 bytes and one "tape" or folder containing 64 images occupies 32 Mbyte. One CD holds up to 20 "tapes" worth of data. If you do not have Ethernet, we can send your images on CD.

STEM images consist of a 4096 byte header followed by 512x512 pixels of image data consisting of two channels (8-bit each) interleaved. PCMass25 "reads" a STEM image, displaying both image channels side by side with the header information below the left image. The program also "recognizes" the magnification and sets the calibration automatically. Any image or portion of the display screen can be copied to the clipboard and pasted into a document or image, as will be described below. The current version of PCMass does not print directly and has limited image filtering and annotation capabilities, so a second standard image package such as PhotoShop, Canvas or Paint is recommended for publication quality output. Similarly, graphs and histograms are intended to give a rapid view of the data in hand and publication quality can be obtained by importing results files into SigmaPlot or similar software.

STEM images can be displayed, annotated and printed with PhotoShop using the "Open As" and "RAW" import mode in the "File" menu with the above parameters entered into the specified fields (512, 512, 2 channels, interleaved, 4096 header). Some care is required to determine which image is the large angle detector signal (the channel most reliable for mass measurements). PhotoShop also permits saving a "flattened" image (use the "flatten" option in the "Layers" Menu) as a .jpg or .tif file using the "save as" option for distribution to other programs or users. This allows image compression to save space.

Data in the STEM image comes from 3 detectors: LA (large angle) & SA (small angle) dark-field annular detectors (40-200 & 15-40 mRadian acceptance angles, respectively) and a

bright-field detector (BF, 0-15 mRad). All detectors are scintillator-photomultiplier with nearly quantum efficiency. For thin specimens, both LA and SA signals are proportional to the number of atoms weighted by Z (atomic number) in the irradiated pixel. This is the basis for STEM mass measurement. The BF signal is 1-(LA+SA), so only two signals give independent information. Normally LA & SA are recorded but for thicker specimens, such as those embedded in a matrix (e.g. stain), BF & LA may convey more information. Details of small corrections applied during signal read-in are discussed below.

The key to reliable STEM mass measurement is accurate background determination. With even the thinnest carbon film substrate, the particle is usually less than half the mass (particle + background) within the measuring area. Two issues arise in background determination: 1) deciding which pixels are far enough away from an object to be considered background and 2) deciding whether the background measured away from the particle is valid underneath the particle. Issue 2 becomes critical if the sample contains detergent, salt, sugar, denatured protein or any material other than the specimen of interest. PCMass25 contains a background program, originally written by J. Hainfeld with improvements by T. Baker, which masks out particles efficiently as long as the specimen is not too crowded or contaminated. Several additional means are provided to test the reliability of the background determination, and will be discussed below.

Actual mass measurement consists of selecting suitable particles with an adjustable circle or rectangle. A mouse positions the circle or rectangle and keystrokes adjust size and rectangle length. The program sums intensity minus background for each pixel inside the boundary, then multiplies by the STEM calibration constant. In PCMass25, the displayed mass value is updated as parameters are changed. A mouse-click or '=' keystroke marks the particle on the display and saves the measurement parameters. Showing the mass value places the burden on the user to be objective in particle selection, rather than excluding particles simply because they "spoil" the SD. This is a deliberate decision, since focusing special attention on deviant particles is important to improving future preparations and understanding the specimen as well as its limits of interpretation.

TMV is included in nearly all STEM samples as an internal control. In the past, we used a TMV calibration for each image. However, extensive measurements have demonstrated that the STEM mass calibration is very stable and fluctuations in TMV M/L (mass per unit length) are primarily due to variation in specimen quality. Therefore PCMass25 uses a standard STEM calibration value (115 Dalton/intensity unit with 1 nm pixels) instead of a TMV calibration. If extensive measurements suggest that a different TMV value should be used, we advise scaling the data in your plotting program. It is always a good idea to check all unobstructed TMV segments for M/L and shape as well as examining the squareness of ends (most sensitive to damage). Many problems which could compromise data quality can be sorted out using the known TMV structure.

A number of models are provided as an aid in selecting particles of certain types. PCMass 25 automatically adjusts the size, amplitude and orientation of the selected model, displaying image, model and difference image in separate windows. Rotational power spectrum, radial mass profile and density profile are also displayed. The difference image can be especially useful in identifying damaged particles or salt. Separate mass statistics are maintained for measurements made relative to each of the 80 available models. Extensive capabilities are provided for viewing mass measurements and histograms of single images and groups of images.

Once a suitable set of models is defined, particles can be selected automatically on single images or groups of images. This has the advantages of speed and objectivity, although there are pitfalls. With manual particle selection, even an experienced user will tend to select a different set of particles in a repeat series of measurements.

New Features in PCMass25: 1) on-screen help, 2) profile & power spectrum along a line, 3) subtraction mode for mass measurement and review, 4) correction of conflicts with Windows XP.

2. Installation

Please note that this program is evolving rapidly. It replaces the DOS version (PCMass15) which was unsatisfactory in display quality and some technical points. Certain combinations of operations can hang the program. If this happens, the best choice is to hold down ctrl and alt while striking del, terminate the job and restart the program. PCMass25 also contains features under development and not documented here which can give unexpected displays if activated by accident. If you have questions, please note the sequence of keystrokes leading to the problem and call Joe Wall at 631-344-2912. If you can call from a telephone next your computer and have the program running, this will expedite communication and troubleshooting.

To install the program:

a. Create one main folder and three sub-folders:
C:\PCMass25 (any drive letter can be used)
C:\PCMass25\MassMeas\
C:\PCMass25\SpecAves\
C:\PCMass25\nk7375\.

b. Copy the following files into your C:\PCMass25\ folder:
PCMass25.exe
PCMmods.dat
PCMass25.doc

c. Copy the supplied practice images of TMV and earthworm hemoglobin in the nk7375 folder into your C:\PCMass25\nk7375\ folder. Output from the analysis of single images will be saved in the C:\PCMass25\MassMeas\ folder as nk*.smm (STEM Mass Measurements, * = the nk number of the current image). These are actually text files which can be read by any editor program or imported into plotting programs. Averages and histograms for multiple files will be saved in files named for the first specimen in the series and placed in the C:\PCMass25\SpecAves\ folder as text files.

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3. File Import to Your PC

STEM images may be brought to your computer on a CD (650 MBytes, holding up to 20 folders ("tapes"), each containing 64 image files and one directory, for a total of 1280 images), on a floppy disk (holding 2 image files) or by e-mail transfer. FTP is again possible using our server:

ftp.stem.bnl.gov. Detailed instructions are available as a separate document and Tutorial. When you have files to download, we will contact you and put your images into the Pub directory on our FTP server. When you confirm the download, we will erase them.

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4. Checking Your Computer's Configuration

In order to see the entire image with the best detail, your display should be set to 1024x768, true-color or high-color. If your display is smaller, information will be lost and if it is larger (e.g. 1280x1024) the images will not fill the entire screen. If the color mode is lower, only limited gray scales will be available, similar to PCMass15. Check the display mode by clicking on Start (lower left corner of screen) and selecting "settings" and "control panel". Double click on the "Display" icon. A set of tabs should appear along the top of the panel. Select the one on the right, labeled "settings". Drag the "Screen Area" slider right or left until 1024x768 appears. Click the pull-down arrow under "Colors" and select true-color or high-color. Then click "Apply" at the bottom right. If the display fails to switch to the correct mode, your computer may not have enough display memory. Other windows settings should not matter to the program.

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5. Starting PCMass25 & reading in an Image

Start the program by clicking on the "Start" button in the lower left of the screen. In the space next to "Run", type c:\PCMass25\PCMass25 followed by <Enter>. An easier method is to start Windows Explorer, open the PCMass25 folder and double click on PCMass25.exe (Note that the suffix "exe" may not be displayed unless you turn off the "suppress known file types" option in Windows Explorer). You can also create a shortcut by dragging PCMass25.exe to the desktop. Exit PCMass25 by striking the <Esc> key, by using the "Exit" command in the file menu or holding down <Ctrl> and striking 'x'.

The program should start in 10 sec or less, displaying a practice image of earthworm hemoglobin and TMV from the nk7375 folder. You can select any other image using the "File" pull-down menu (top left of the screen) and selecting STEM_Image. An open-file window should appear showing the contents of the current folder. Navigation is the same as a standard Windows program. Left-clicking on the "Look-in" panel at the upper left of the window shows the directory tree. Navigate by clicking on the folder you want. Click on the file you want to select to place it in the "File Name" panel near the bottom of the window. Click "Open" to load it (or double click on the file name).

Frequently one wants to **step through sequential files**. Advancing to the next file can be done by striking either the '1' key or the '+' key. Go back one file by striking the '-' key. The same thing happens if you click with the mouse on the "hot spots" at the bottom center of the display labeled "Next File" and "Prev File". To skip forward or back exactly 64 files, use the "Next Tape" and "Prev Tape" hot spots. Note that hitting "Next File" when on file 64 goes on to file 1 of the next tape. Similarly in reverse. If the selected image is not present on your a disk, an error message box will appear, (You may need to click on OK several times to get this to go away.)

Normally we collect data in units of six or 64 images. It is useful to know what is in the remainder of the folder. PCMass25 incorporates the old **64-on-a-page** program supplied with

PCMass15. When you move the cursor to the right of the centerline and the program is otherwise idle, it scans through the entire current folder (block of 64 files) making thumbnail images and checking the headers for magnification, new specimen number and presence of previous measurements on each file. The thumbnails are displayed on the right panel of the image whenever the mouse pointer is in the right portion of the screen. The header information for the first image of a new specimen is given at the bottom of that panel, separated from the previous specimen data by a horizontal black line. A bar to the left of the file numbers indicates the scan size of the image (magnification increasing to the right). The image being viewed has a black or partially black bar. Images with measurements already in the "MassMeas" folder have purple bars and the remainder are green.

Moving the mouse pointer over the thumbnail images or up and down in the lower right panel causes one file to be selected as denoted by a white box around its thumbnail image in the upper right panel. The full header of the selected file is displayed in the bottom center. Clicking the left mouse button causes the highlighted file to be read in. This mode is useful for a quick survey to see similarities and differences among specimens in an experiment.

Prior to image read-in the program draws several curves (see below) for data linearization. Then it writes the channel 0 image (usually large angle annular detector, LA) to the left panel and the channel 1 image (usually SA) to the right panel and displays the header under the left image. If the **contrast** is not suitable, run the background as described below, place the mouse pointer on an object you wish to be white and strike the 'k' key. The background program provides a reliable "black" level and removes the effects of sloping background. This can also be done with Photoshop.

Linearization of the detector signals is more important for thicker specimens. The bright field (BF) signal obeys Beer's law falling monotonically to zero exponentially with a characteristic mean free path. The LA and SA signals are more complicated, initially increasing linearly, peaking and then falling back to zero for very thick specimens. This can be described by the equations:

$I_{BF}=I_0*\exp(-t/1100)$
$I_{SA}=I_0*(1-\exp(-t/2500))*\exp(-t/2000)$
$I_{LA}=I_0*(1-\exp(-t/1964))*\exp(-t/10000)$

where I_0 is the incident current, the incident voltage is 40keV, I_{BF}, I_{SA} and I_{LA} are the measured currents on the bright field, small angle and large angle detectors, respectively and t is the total specimen thickness in A (assumed to have density = 1) along the direction of the beam. For thin specimens, the exponentials can be approximated, giving a linear relationship between annular detector current and specimen thickness. At any point in the thickness curve the combination of signals giving the best S/N can be calculated from counting statistics. The STEM1 computer system now in use records 2 signals simultaneously. For thin specimens, LA and SA are recorded after normalizing by the sum of LA+SA+BF. For thicker specimens, LA and BF are recorded, either normalized or direct. For thin specimens, LA & SA detector preamps are set to a gain of 10 to make best use of the 256 available recorded gray scales. For thicker specimens, gains of 5, 2 and 1 may be used.

PCMass25 corrects for deviation from a perfect linear relationship by using a look-up table to read in the LA, SA and BF signals. After starting the image read-in procedure, it first plots the

above curves appropriate for the detector gains (see below) with LA in purple and SA in yellow. Also plotted is the look-up table to be used with the input data value along the X-axis and the "corrected" value in the Y direction. For a specimen like earthworm hemoglobin, the correction at the thickest portion of the molecule is roughly 5% for the LA detector and 11% for SA.

If any STEM **detector gains** or **pixel size** are not correct, they can be changed by clicking on the appropriate hot-spot at the bottom left of the display. Normally the read-in program picks up the pixel size from the image header (scan size). The detector gains are normally 10 for LA and SA. However, for thick specimens lower gains (5,5 or 2,2) are used and these must be entered. The STEM mass calibration is adjusted for the detector gain & pixel size and is normally stable over time (115.0 Dalton/intensity unit). If the preamp gain was not 10 when the image was recorded, this should become obvious as soon as you start measuring TMV M/L. Instead of 13.1 kDa/A, you will get 6.6 (if G = 5) or 2.6 (if G = 2).

In the case of negatively stained specimens, we normally record the BF signal on Ch1, since the SA signal is relatively flat around 1,000A apparent thickness typical of good stain areas and therefore contains little information. PCMass25 searches the header for terms such as uranyl or vanadate indicative of negative stain. If it finds one, it assumes that Ch1 contains the BF signal and Ch0 is the LA signal, setting the preamp gains to LA=2, SA=0 and BF=1. The maximum information will then be in the difference BF-LA which it places in the Ch0 display. Such an image is useful for viewing or comparison to models but not for mass measurement, except in special circumstances.

The **size of a pixel** is read by the program from the image header and displayed along the bottom left of the image. A 0.512u scan with 512 pixels has a pixel size of 10A or 1 nm. If this is incorrect for some reason, enter the correct value by clicking the mouse on the appropriate area. Note that the pixel size is an important parameter in mass measurement.

Simple **length measurements** can be made on the upper left image as follows. Place the mouse cursor on the starting point and hold down the left button while dragging the pointer to the ending point. This will draw a line on the screen and print the end-to-end distance (temporarily). The X,Y coordinate of the mouse pointer and the values of ch0 and ch1 for that pixel are displayed near the bottom center of the screen. The profile along the line (plus 10% beyond the endpoints) will be displayed below the image. A pair of parallel lines superimposed on the image encloses the pixels contributing to the profile (change width with t/y).

A **power spectrum** of frequencies along the line defined above and within the indicated region appears below the profile. The most prominent periodicity is identified and printed below the spectrum. Note that the starting and end points should have approximately the same intensity, otherwise the signal will have a sloping baseline and the longest period component will be dominant. The bands displayed on the right panel are: (closest to the center) a sine (cosine) wave with the fitted amplitude and phase and (on the right) the original image data minus the displayed periodic intensity. The lowest frequency is at the top and each trace has one additional harmonic period. If the endpoints of a periodic object are not in equivalent points in the waveform, the intensity will be split among nearby frequencies. Therefore it is useful to move the endpoint controlled by the mouse and observe the changes in power spectrum to get the highest intensity in a single order. The actual amplitudes and relative phase angles are printed to the right of the image

slices. Clear the right portion of the screen by briefly moving the mouse to the right of the centerline and back to the left.

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6. Computation of Background Signal Level

The second item in the main menu along the top edge of the image is Background computation. Many features of PCMass25 will only run if the BKG_VALID flag is true for the current image. Attempting a mass measurement before the background is computed will activate the background program automatically. A red message in the center of the left image reminds you to run the background routine. See discussion above regarding the importance of a reliable background value.

To run the background program, strike the '2' key to use default values or use the pull-down "Background" menu for other options (see below). A mask is displayed which should blue-out all particles and TMV in the image. The background in the remaining image, as well as the standard deviation (SD) and number of non-masked points is printed for 16 sub-areas (4 X 4, each containing 16,384 points). The left table is for Ch0 (LA) and the right is for Ch1 (usually SA). Below these tables are averages for the various parameters and an estimate of the dose used in recording the image. The intensity distributions on the Ch0 and Ch1 images are displayed following background computation. Note that the plot is semi-log and some intensity values may be missing due to the action of the look-up table during read-in. Moving the mouse will restore the image.

The first thing to note is whether or not enough image was masked (some particles not completely masked) or too much was masked (leaving too few points for valid determination). If the sample is very crowded, this may be a serious problem. If the number of points used (out of 16,384) drops below 1,000, extreme caution is advised. If it is not obvious whether all the proper points are being masked, try the "Show Mask" option in the Background menu. This will redisplay points outside the mask in high contrast (yellow) and points inside in normal contrast so you can see if particle edges are being masked properly.

The threshold for masking affects the number of points masked. Normally we use 3.0 standard deviations above the mean. Raising the threshold will exclude less background and lowering it will exclude more. Values of 2.0, 2.5, 3.0, 3.5 and 4.0 are options in the "Background" menu. A typical thin carbon film should give a signal value between 20 and 30 with a preamp gain of 10. The program assumes that pixel values of zero are holes and masks them also. Please note that the count on a hole was 11.5 for older files instead of zero, so detector offset could be checked. New voltage to frequency (V/F) converters eliminated the need for this offset and it has been eliminated. PCMass25 subtracts this offset from older files on read-in (Files before nk810912).

In some cases, some of the 16 sub-areas may be totally covered, either by a large object or overcrowding of particles. Background values in such areas will be printed in red and will be replaced by the global average. If none of the 16 sub-areas gives valid background, zeros will be entered for all areas and the BKG_VALID flag will not be set.

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7. Manual Mass Measurement

PCMass25 uses a circle or rectangle positioned by the mouse to delineate pixels for mass measurement. The mass values and pixel coordinates are displayed every time the mouse is moved or the size is changed. Three values are determined: mass using picture background (see above), mass using local background (see below) and mass of a fitted model. The "Mass" menu has options for circle measurements (default on startup) or rectangle measurements. Two types of rectangle measurements are possible: **mass per unit length** (total mass in rectangle divided by rectangle length) and total mass within the rectangle. The "chain M/L" option chains rectangles and sums the lengths to give the total length. A new option gives **mass per unit area** (in Da/A**2), either for circles or rectangles. The **radius** of the circle or **half-width** of the rectangle is changed by 't'/'y' keystrokes, with 'y' increasing the current value by 1 pixel. The **rectangle length** is changed by ';'/'l' (semicolon) keystrokes with 'l' lengthening the rectangle. The program "guesses" the best rectangle orientation from the power spectrum and from fitting the selected model [Note: bkg must be valid for this feature]. If this is not close enough, the rectangle can be **rotated** by 'z"Z'/'x"X' keystrokes, with the capitals moving in 10 degree increments. The current radius or rectangle half-width is displayed to the right of the zoomed model as R=xxx and the current rectangle length is displayed on the same line. If any portion of the measuring area goes off the edge of the image, the mass will be set to zero, but the analysis can be saved. Measurements with zero mass are excluded from averages.

Local background is measured as follows. In the circle mode, three concentric circles are drawn. The pixels within the center circle are summed for the mass measurement. Pixels outside circle 2 (5 pixels larger than circle 1) and inside circle 3 (twice the radius of circle 1) are used to compute the local background. Note that the background mask is retained and only unmasked points are used for local background. As you move the mouse, unmasked pixels between the outermost two circles appear with normal contrast while masked points change to white. Excluding these points may result in no useable pixels, in which case the picture background is used for L_Mass also. In rectangle mass, larger concentric rectangles are drawn, similar to the circles discussed above. For rectangle M/L (mass per unit length) mode bands are drawn on both sides of the measuring rectangle and the local background computed in the outer bands on either side of the centerline. Using the background mask makes it possible to get a useable local background even in crowded areas.

The mass using the **Picture background**, P_Mass or Mp, is generally more reliable and gives a better standard deviation (SD) than the mass computed using the local background. L_Mass or Ml may be interesting in special cases and any significant difference between L_Mass and P_Mass is a sign of trouble in the measurements. Model mass will be discussed below.

Make a **mass measurement** by positioning the circle or rectangle over a particle. The contents of the measuring circle or rectangle are zoomed or de-zoomed to fill a viewing window of 80x80 pixels below and near the right edge of the Ch0 image. The P_Mass or P_M/L is displayed immediately under the zoom window and the L_Mass or L_M/L on the next line down. At this point it is instructive to vary the radius of integration (t/y) and observe how the mass value changes. If the radius is too small, the measured mass will drop. As the radius is increased from a too low value, the mass should increase, then plateau, and finally begin to diverge as extra background noise is included. Move to "clean" background areas and note the fluctuation in mass from one area to another (should be symmetrical above and below zero).

Record a mass measurement by centering the measuring area and pressing the left mouse button or striking the '=' or <ENTER> key (slower but less likely to move the center point). The measurement is entered when the button is released. If the mouse moves upon release, the measurement will not be entered. Try again. "Fine tune" the centering by leaving the mouse fixed and moving in single pixel increments with the arrow keys. A circle, rectangle or pair of lines should be drawn around the particle when it is entered and the sequential measurement number should appear next to the particle, followed by the value of P_Mass. The measurement parameters and mass values are saved in a buffer, which is written to disk when a new image is read or the program is shut down. The LA particle image is also copied to the right screen, along with the reference model (see below), difference image with superimposed rotational power spectrum, SA particle image and LA minus SA image (may be non-zero on salt contaminants). Particles can be **deleted** starting with the one most recently entered by striking the 'Backspace' key.

A **reference model** is displayed in the small panel below and to the right of the zoomed measuring area. This serves several convenient purposes. Mass measurements are labeled with a "reference model" number and 80 separate running averages are maintained. This permits measuring different types of particles in any order without scrambling the averages. One of the 80 available models may be a reasonable approximation to the size and shape of your object, so it provides a convenient visual reminder of which type of particle you are aiming for. Each has a pre-programmed radius of integration, length, size, shape and name. Selecting that model loads the respective parameters. To select a model, choose "select" from the "Model" menu or click the mouse on the model display panel (bottom center). Move the mouse until the model of interest is highlighted and press the left mouse button. Repeat this until you find the one you want and adjust radius of integration etc. to fit your particles.

The area to the right of the zoomed image and above the zoomed model displays the **difference image**. A search routine adjusts the model parameters to minimize the RMS (root mean square) difference between model and image pixels. The numerical results of the optimization are printed to the right of the difference image and the parameters describing the quality of fit are printed below the mass measurements. Any parameters outside preset limits are printed on a **red** background. DX and DY are displacement in pixels from center pointed to by the mouse. T is the rotation angle of the model. SZ is model zoom giving the best fit and HT is the relative signal amplitude. The quality-of-fit parameters are normalized so that a perfect fit would give a value of 1.0. Those three parameters are: 1) brms, the RMS deviation from background of pixels between the inner circle and the middle circle (or rectangle), 2) rms, the RMS of image minus best-fit model for all pixels within the innermost circle and 3) srms, a comparison of rms values with the model at the best rotation angle versus that with the model at the worst rotation angle.

The upper and lower cutoffs for each selection parameter are given in the model definitions. For advanced users these reference particles can be used for automatic particle selection and alignment, as in PCMass15. Models are defined in the PCMmods.dat file supplied and are easy to customize as described later. The color of the particle #:Pmass value printed next to the particle is keyed to the current reference model #. Note that the measuring and model windows both zoom as the radius/length of integration are changed. Note also that a **rotational power spectrum** is displayed at the bottom of the difference image, indicating the most prominent symmetry of the particle in the viewing window [Not available until bkg is computed].

The "best" center of the particle as found by the refinement program is printed below the srms parameter. The coordinates are in pixels relative to the lower left corner of the image. This is the first quadrant of a Cartesian coordinate system and angle increases counter clockwise. Note that the computer screen coordinate system has 0,0 in the upper left corner and this may lead to some confusion; it certainly is an annoyance in programming. The starting center is the sum of the mouse X,Y printed below header plus the keyboard offset from the arrow keys, printed above the mouse X.Y plus the DX,DY values determined by fitting. The image intensities on channels 1 & 2 are printed after the mouse X,Y. You can check the intensities of neighboring pixels using the arrow keys. However the range is only +/- 3 pixels. The XKB and YKB values are reset to zero by a mouse move greater than the radius of the measuring circle.

After reading in image c:\nk7375\nk737537, running the background program, selecting model 50 (default), and moving the mouse to X=299, Y=60, the bottom center of the screen should present the following information:

|**********||**********|		**DX**=0.270	model X offset in pixels
| Zoomed |	| Zoomed |	**DY**=0.318	model Y offset
| Image |	| Difference |	**T**= 14.104	model rotation angle
|	| Image |	**SZ**=1.008	model zoom
|	|	**HT**=1.013	model relative intensity
**********	********** |	**XT**=0.000	model tilt (3D only)
Mp=3.660	| Zoomed |	**YT**=0.000	model tilt
Ml=3.695	| Model |	**R**=16 **L**=0	radius of integration, rectangle length
brms=0.743	|		(in pixels)
rms=0.641	|		
srms=0.327	**********		
299.3, 60.3	50 Ehb face	Model X,Y	model number and name
	Mm=3.466		model mass after fitting

[Note, entries on a **red** background are outside selection parameters for that model]
Mp = particle mass or M/L using global (picture) background
Ml = " " " " " local background
brms = RMS deviation from background for pixels between circle 1 and circle 2
rms = RMS of image minus model for pixels inside circle 1
srms = a measure of the best fit versus that with the model rotated to the worst position
X,Y is the fitted center in pixels relative to the lower left corner of the screen

Depending on the speed of your computer, you may notice a delay in completing this information. To be compatible with Windows multitasking, PCMass25 processes the image in short bursts. The first priority is moving the mouse pointer (arrow) in response to mouse movements. The next operation is filling the image zoom window, followed by drawing circles or rectangles, fitting the model, redrawing if necessary, then filling the zoomed model and difference image windows. Finally, the program checks for previous measurements near that point with the selected model, printing the relevant parameters to the left of the zoomed image area. If the mouse is moved at any time the sequence is aborted and starts over at the new image point. The arrow keys allow fine-tuning the mouse pointer +/- 3 pixels in any direction.

Any delay caused by model fitting is compensated for by elimination of the need for rotational alignment of rectangles (e.g. TMV, model 21). The model zoom parameter, **SZ**, gives a quick measure of relative size. If the model is a reasonable approximation of your object, the difference image highlights imperfections or accretions. The **fitting process** can be viewed at several different levels using the DISPFLAG parameter at the bottom left of the screen. Normally this is set to zero for the fastest operation. Increasing the level increases the detail of the displays provided. Many of the displays in levels 3 and 4 pause after certain operations (to allow careful study) until you move the mouse.

The value of **picture background** is obtained by bilinear interpolation from the 16 values calculated by the background program. The **dose** is calculated from the background standard deviation and absolute intensity, assuming this is mainly due to counting statistics. **brms** is computed in the 5 pixel wide swatch just outside the radius of integration and is the ratio of bkgSD from the background computation divided by root mean square (intensity minus bkg). For clean carbon, this should be 1.0. If the radius of integration is too small or there is dirt around the particle, the value of brms will decrease rapidly. A value less than 0.5 is a sign of serious problems with that measurement. If the background has not been run, zero will be printed for this parameter.

The absolute value of the **rms** parameter is normalized relative to the dose and average signal within the measuring area and should be close to 1.0 for particles which can be fit well by the model. If the background has not been run, zero will be printed for this parameter.

The absolute value of the **srms** parameter is rms(aligned)/{rms(aligned)+rms(rotated)}. The rotation angle is half the rotational symmetry angle of the current model. For objects with large rotational modulation such as TMV, values of srms of 0.9 or greater are possible (90 deg rotoation). Typical earthworm hemoglobin values are 0.4 (30 deg rotation).

An interesting exercise is to move the measuring circle or rectangle over clean background and particles, noting the variation in mass values. Then center on a particle and vary the radius of integration ('y'/'t'). If the radius is too small, part of the mass will be missed and if it is too large, the value will fluctuate over an increasingly wide range as a larger background mass (as well as stray objects) is integrated, then subtracted out imperfectly. Proper choice of the radius of integration is essential to good mass values.

The panel to the left of the zoomed image displays the **projected mass profile**. For filaments, this is the average signal as a function of distance from the centerline and for round particles it is the average signal as a function of radius from the center of the zoom window. The blue curve uses large angle data and green is small angle. The red curve is the apparent density of a cylindrically or spherically symmetric which would give the observed projection. Note that left and right halves of the curve are computed independently so asymmetry and noise are more evident. Note also that for round particles the center of the curve is noisy due to the smaller number of pixels contributing to the average.

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8. Viewing Results & Statistics

Each mass measurement ('=', <ENTER> or left mouse button) makes an entry in memory consisting of: type of measurement (MMM in this case for manual mass measurement), particle #, model # (label), rotational symmetry, radius of integration, length of rectangle (0 for circle),

measuring mode ('3', '4', '5' or '6'), L_Mass, P_Mass, M_Mass, x position of center of circle or rectangle, y position, angle in image plane, tilt angle & rotation about Z axis (for 3-D models only), size, height, stretch (not used at present), picture background, brms, rms and srms (see above) . Up to 1,000 measurements per image can be accommodated.

Mass measurements for an image are saved in c:\PCMass25\MassMeas\nkxxxxyy.smm when the image is closed (next image read or program terminated). This file can be opened with Notepad (the Windows accessory) or any text editor. Be sure to choose a font without proportional spacing to keep the columns straight. To import this into SigmaPlot or other plotting package, copy the .smm to a second file and trim off the extra descriptive information.

When an image is read into PCMass25, the program checks for a corresponding c:\PCMass25\MassMeas\nkxxxxyy.smm file and reads it into the results buffer. All subsequent measurements are appended to the same file. If you instead want to start fresh, use the "Erase_smm" option in the "File" menu. This erases the previous *.smm file as well as all the previous measurements. To delete the entries individually from the present session, starting with the most recent, strike the <Backspace> key.

Each stroke of the **'e'** key **displays** 40 entries from the **current results file**. If there are no more entries, the display resets to the first entry. You can also check individual particles by placing the mouse pointer close to the particle center. If a measurement has already been made on that particle with any model, it will be printed to the left of the present measurement, indicating the model # used, the sequential # and the values obtained. Note that this will obscure part of the profile display.

The **'r'** key or "This File" option in the "Stats" pull-down menu provides a **synopsis of what has been done on the current file**. This starts with a display in the lower portion of the screen with one column for each of the first 5 models used. Underneath the model # and name are nine entries consisting of mean, SD in %, number of measurements. There are three types of measurement: M for manual, T for trial and R for R-align (see description below of automated selection, 'T' & automated alignment, 'R'). Each type has three levels of testing: all particles of that type, only those passing the bkgRMS (brms) test for that model and those passing all tests for the model. Use the arrow keys to hi-light the measurement of interest. At the bottom of each column are compact histograms for each model with passing particles in green and failing in red. The right panel should show an expanded histogram for measurements in the highlighted category. Superimposed on the histogram is a Gaussian with the same mean and standard deviation.

To see which **particles** are **in various bins** in the histogram, hold down the <SHIFT> key and position the mouse below the expanded histogram. All particles in the selected class will be marked in red on the original image (left panel) and those with mass values in the bin pointed to by the mouse will switch to white. Move the pointer back and forth under the peak to see where individual particles are highlighted.

Above the expanded histogram are scatter-grams for various image and fitting parameters. The purpose of this display is to highlight any **systematic errors** in mass measurement due to specimen imperfections or poor choice of selection parameters. The first horizontal row immediately above the expanded histogram compares (from left to right) Ml, Mm, X-coordinate, Y-coordinate and bkg. plotted vertically versus Mp plotted horizontally. The individual measurement points are color coded as follows: GREEN = particles passing all tests, YELLOW =

particles passing bkgRMS test but failing some other test and RED = particles failing bkgRMS and at least one other test. A straight line with a slope of 45 deg. indicates a high correlation between Mp and the particular parameter. The left panel, Ml vs. Mp, should show nearly perfect correlation if the measurements were done correctly, with slightly higher errors in the Ml measurements, as discussed previously. The next panel to the right, Mm vs. Mp should show a high correlation if the chosen model is a reasonable approximation to the particle of interest and the fitting is working correctly. The next three panels to the right should show no correlation between Mp and X position of the particle, Y position or background value. Any suggestion of systematic errors should be investigated, since averaging many particles will not remove such errors.

Above the expanded histogram and the first scatter-grams is a second set showing correlation between Mp and the various parameters from the model fitting process: brms, rms, srms, size and height, with the selection "hurdles" indicated by dotted red lines. The dotted white line indicates a value of 1.0. In each case **valid measurements** should cluster and be well separated from measurements on problem particles and well above the respective "hurdles". The selection parameters can be changed by holding down the <SHIFT> key and dragging the appropriate line with the mouse pointer. (To see the effect on the histogram, strike the 'r' key twice.) Selection parameters can also be changed permanently by editing the PCMmods.dat file and striking the 'M' key (reload models) as described previously. Proper choice of model parameters should give both size and height scatter-grams centered vertically near 1.0 (dotted white line) in their respective windows. If this is not the case, you can try a different model or adjust parameters on the selected model as described below.

Clear the screen at any time by moving the mouse to go back to a normal screen. Restore the screen to **remove all overlays** by striking '9'. **Save the screen** to the clipboard using <Alt><Print Screen> and paste into a PhotoShop document for printing.

Strike <ENTER> to redisplay the image with particles/segments of the highlighted type marked. Hold down <Shift> while pressing <Enter> to **print particle number and mass** next to each particle.

Strike 'D' to **image-average** particles entered using the current model and passing all selection parameters for that model. LA and SA images of the selected particles before rotation are displayed in a **gallery** in the right image. The left image shows expanded images of: LA image, rotated, translated and averaged (top left), 0.9*SA image (bottom left), LA minus 0.9*SA (top right) and LA minus model (bottom right). To add to a previous average, use 'd' instead of 'D'.

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9. Model Selection and Tune-up

Space is provided for 79 model definitions. To select a model, choose "select" from the "Model" menu, hit the 'm' key or click the mouse on the model display panel (bottom center). Move the mouse until the model of interest is highlighted and press the left mouse button. Repeat this until you find the one you want and adjust radius of integration etc. to fit your particles. Models are made up of hard-edge or Gaussian balls or cylinders which can overlap or interpenetrate in 2 or 3 dimensions. Some 3-D models are included in PCMmods.dat, but use of these is not fully implemented in PCMass25 and will not be discussed further. Models are useful for visual reference, automated particle selection, alignment and image averaging. Fitted size and amplitude provide a convenient way to compare particles in a set. The quality-of-fit parameter, RMS difference between image and model, may be useful in judging particle quality. This is

printed under the zoomed image. The actual value printed is the background RMS for the entire image (from the background determination) divided by the particle RMS. This ratio has the advantage that it is essentially independent of dose and approaches a value of 1.0 for a perfect fit to the model.

Since model customization may not be straightforward, J. Wall will be happy to provide a PCMmods.dat file with starting models suitable for your situation. These can be fine-tuned as described below.

Each model has associated a name and a set of selection parameters, upper and lower limits for each fitting parameter. Model information is stored in a file named PCMmods.dat. One way to "tune up" a model is to start with one which is similar to your structure and edit the PCMmods.dat file with Notepad (double click on the file name in Windows Explorer). Each line recognized by PCMass25 begins with a key word: MODEL, BALL, SELL, SELH and NAME. Other lines are ignored so comments can be interspersed. Follow the instructions at the beginning of the file. Make a copy of the original before editing in case something goes wrong.

Close the edited PCMods.dat file and initialize all models to file values by striking the 'M' key. Compare the model to your particles by re-selecting it and placing the mouse cursor over a "good" or "bad" particle. The image pixels should appear zoomed with its Mp below and its Ml (mass using local background) on the following line. The RMS difference between image and model pixels is displayed below the zoomed image and the next line shows the brms from ring or rectangle surrounding the particle. The arrow keys step the image one pixel at a time. Adjust integration radius with tT/yY and the particle angle with zZ/xX and observe the change in RMS and difference image. For 3-D models, Qq/Ww tilts about the X axis and Aa/Ss rotates about the Z axis. Note that pressing the left mouse button or the <ENTER> key will save the current parameters (for the particle pointed to by the mouse) in the *.smm results file.

A quick way to optimize a model is to select a model with the desired symmetry and degree of complexity (number of balls), choose a "good" particle in the field of view and place the mouse cursor on it. Check the size and ht parameters and the quality of fit. If the rms is greater that 0.1 and the absolute values of DX & DY are less than 1.0, the fitting routine probably "locked" onto your particle. Vary the radius of integration (t/y) so the particle you have chosen is fully inside. Note the values of SZ and HT to the right of the difference image. If they are not both close to one, strike F9 (the F keys are along the top edge of the keyboard) several times to scale the entire model so that SZ and HT are both close to 1.0 after fitting. Then strike F4, F5, F6, F7 and F8 to adjust ball sizes and positions individually. Each F key adjusts the parameter it controls +/- one or two small increments for each ball in the model (up to 24 balls). The RMS value for the starting position is shown in the center of the 5 columns displayed, with RMS values for increments of that parameter displayed to the left and right. Any improved RMS values are highlighted. The one increment making the greatest improvement in RMS is retained. When no further improvement is obtained by striking any of F4-F8, the process is complete. Note that this may not be the optimum model obtainable with this number of parameters because the small +/- increments may become stuck in a local minimum, rather than the global minimum. If the appearance of the difference image suggests that a better model is possible, strike 'M' to revert to the default definitions of all the models in the PCMmods.dat file and try again using a different sequence of increments. When you are satisfied, note the final parameters and put them into PCMmods.dat with Notepad. For an unknown structure it is useful to try several different models for the same type of particle.

10. Automatic Particle Selection and Alignment

If you re-measure a set of files, would you pick the same particles and get the same answer? The main motivation for automatic particle selection is to get around this dilemma. Practical benefits include speed, reduction in tedium and potential for image averaging. However, the pitfalls are plentiful so careful manual analysis is essential before starting and thorough review is essential before concluding.

Start the procedure by selecting one of the models you wish to use and tuning it up as described above. Make one or more manual mass measurements using that model. Repeat this for all models of interest. Be sure to include TMV (model 21) as one of the models. Basically you are "teaching" the program what to look for. Strike the 'r' key to display the results to date. This enters all the displayed models into the search list. Select "Delete *.smm" in the "File" menu to clear all previous measurements. Next strike F11 to activate the automatic search for all selected models. This masks the image at a threshold proportional to the height of the selected model above the background and traces around the outline of all isolated patches, determining maximum and minimum radius from the center. Particles approximating the size of the search model are marked with a circle or rectangle and entered in the *.smm list with category 'T' for trial. This procedure repeats for all models selected. If some particles were missed, you can enter them manually.

Clear the screen by striking the '9' key. Then hit F12 to initiate model fitting. Again, particles passing the various tests will be indicated with a circle or rectangle and entered in the *.smm list, now with the category 'R' for realigned. This normally takes somewhat longer and some particles may be picked up by more than one model. Save the results by selecting "Save *.smm" in the "File" menu. Review by striking the 'r' key again and move the highlight with the arrow keys.

If you are satisfied with the performance on a single file, you can extend the measurements to a **block of contiguous files** as follows. Move the mouse cursor to the small green bar of the last file in the sequence (bottom right panel). Its header should be displayed to the left of the green bars. Hold down the left mouse button and drag the mouse pointer to the magnification bar of the first image you want and release the left mouse button. All the bars of files of interest should be enclosed in the displayed rectangle and that starting & ending file numbers displayed. If this is not what you wanted, repeat the procedure. Images with the desired magnification values (inside the rectangle) will be analyzed when you hold down the <Shift> key and strike F11. The program will cycle through the entire procedure for each image, including the F12 alignment step, with no further prompting. Depending on the number, size and complexity of the models and the speed of the computer, this may take 1-10 min per image and can be left to run unattended. The screen display gives some idea of progress, although this needs additional work. Note that the measurements for each image are saved in its respective *.smm file, so the automated analysis can include all specimens in a particular folder. Note also that the old *.smm results files are erased in this process, so if you wish to keep them you should rename them or move them to a different folder. The automated analysis run can be aborted by striking the <Tab> key if it is clear that there is a problem. If any portion of the measuring area goes off the edge of the image, the mass for that particle will be set to zero, but the other parameters will be saved. Measurements with zero mass are excluded from averages, although they are shown in histograms.

The automated results for an individual file can be **viewed** by reading in the desired image and striking 'r'. Special care should be given to particles selected by more than one model, since they may be counted twice in some histogram protocols. If this is a problem, the models can sometimes be adjusted slightly to reduce overlap. If you move the mouse cursor to any particle on the left screen which has a previous measurement, the number of the model with the best fit and the fitting parameters will be displayed to the left of the zoomed particle image (overwriting part of the radial profile display). Parameters outside the selection range for that model will be displayed on a red background. It is also a good idea to check the parameter scattergrams on the right screen obtained by striking 'r' then <Enter> for several images. It sometimes happens that the first image in a series or the one used to tune up models is not typical. Most of the "good" particles of each type should be close to the center in each panel and well within the selection ranges, especially the size and height panels. If this is not the case, you may be systematically removing large or small particles from the averages and skewing the results.

Once you are satisfied that most of the particles of interest are being picked up and those failing are being rejected for good reason, you are ready to compute **specimen averages.** This is essentially the same as for the automated analysis, except that the rectangle should be drawn around magnification bars of only one specimen. Strike the 'R' key. The upper left panel will display file number, dose, background and abbreviated histogram for each model. The lower panel will show Ave and SD for each model as in the 'r' mode, but for pooled data. The lower right histogram will show the distribution for measurements of the highlighted type. The data are presented in this way to focus attention on potential flaws in the data set.

First notice any trends in dose or background value and see if they correlate with systematic variation in mass, particularly for the TMV. Mass loss is typically 2.5% at a dose of 10 el/A**2 normally used on a 0.512 micron scan. A 0.256 micron scan at the same beam current would deliver 40 el/A**2 and cause 10% mass loss, so it is usually best not to combine data recorded with different scan sizes without careful consideration. If the specimen contains detergent or salt, the background may fluctuate from image to image and the TMV M/L may fluctuate in the same or the opposite direction depending on the affinity of the contaminant for carbon film or protein. The TMV M/L should be 13.1 kDa/A with a SD of 2% or less under ideal conditions. If the particles are known to have a rigid shape similar to that of the selected model but many are failing the RMS test, the particles may be falling apart. That would also tend to show up as a skew in the overall histogram. For reference in judging this, a Gaussian with the same Mean, SD and integrated area is displayed in the main histogram window.

Each time the 'R' key is pressed a file is written in the c:\PCMass25\SpecAves\ folder containing the Mean, SD and number of passing/number tested for each model and a simple histogram for the highlighted Model and type of measurement.

We have attempted to include enough displays and analysis aids to give confidence in the reliability of the analysis and flag problems where the data or analysis may be flawed. However, we strongly recommend that new users discuss results with members of the STEM Group before publication. We are happy to provide optimized models and sample analysis whenever requested. A particularly effective approach is telephone contact with both parties viewing images of the same data and discussing the finer points of the analysis.

Menus and shortcuts

Main Menu	Shortcut Single Keystroke	Comments
File		
STEM_Image	F2	Normal Windows utility
	1,+,- next image etc. buttons on screen	
Write *.smm		Force an early write
Erase *.smm		Erase previous measurements
Exit	^X, <Esc>	
Background"		
Normal (3.0)	2	
V Low (Cutoff=2.0)		
Low (Cutoff=2.5)"		
High(3.5)"		
V High (Cutoff=4.0)		
Show Mask		
Mass		
Circles		Automatic selection by model
Rectangles		
M/L (see Model, below)		
Chain M/L		
Circle M/A		
Rect M/A		
Reset		Erase entire present series
Model		
Choose	'm', click on model display	
Stats		
This File	'r'	View results for present file
Help		

Other Controls	Comments
<Enter>, =,	
left mouse button	enter a mass measurement
cC/vV	increase/decrease contrast, clear screen markings
bB/nN	increase/decrease brightness, clear screen markings
tT/yY	decrease/increase radius of integration
lL/;:	increase/decrease length of integration
aA/sS	rotate 3-D model about Z axis
qQ/wW	Tilt 3-D model about X axis
<Del>	Remove the last mass entry
'D'	

'e'	list 47 mass measurements
F3	Reset model definitions from disk file
F4	Tune up model- ball distance from center
F5	Tune up model- ball rotation angle
F6	Tune up model- ball Z position (3-D), hole rad. (2-D)
F7	Tune up model- ball radius
F8	Tune up model- ball "density" in arb. units
F9	Tune up model- overall size, combination of F4, F6, F7, F8
F11	Auto-select particles
F12	Auto-align particles

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12. Pitfalls

a. Make sure Caps Lock is not on.

b. If the program does not respond as expected, hold down Ctrl and Alt keys and press Delete. This should allow you to exit and start over.

c. If your model didn't change as expected, did you remember to save the edited PCMMods.dat file, exit the editor and read in the new model info with 'M'?

d. *.smm file deleted with file manager reappears. A copy of *.smm is read into PCMass and writen back to disk when the image file is closed. Make sure the file you are trying to delete is not active in PCMass before deleting. Using the "Erase_smm" option in the "File" menu avoids this.

e. F10 key may lock the keyboard.

f. Windows XT may close the program for no apparent reason after 5-10 min. of operation. Restart and continue where you left off. We are trying to obtain a Windows XT machine to debug this problem. Earlier Windows versions (95, 98, 2000) should be OK.